Synthesis and Characterization of pH and Thermal-sensitive Injectable Hydrogels

Honors Research Thesis

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By

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Abstract

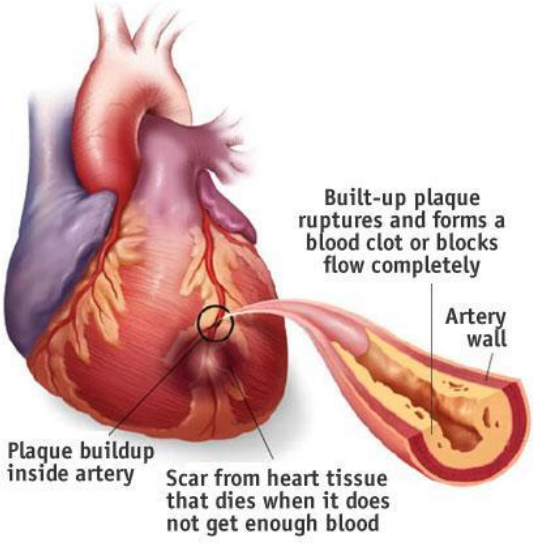
Acute myocardial infarction is a common cause for the significant reduction in delivery of nutrients and oxygen to cardiomyocytes, and with prolonged nutritional absence subsequently propagates chronic heart failure following cardiomyocyte cell death. Biomaterials research retains particular interest to redress chronic heart failure by emphasizing the regeneration of lost functional cardiac tissue via implantation of cardiac stem cells, specifically cardiosphere-derived stem cells (CDCs). While previous research has attempted to elucidate the capacity of novel thermosensitive polymeric hydrogels to sustain CDC survival in the construct, the physical properties of these gels are not well understood. Thus, this study sought to develop and characterize the physical properties of a thermo- and pH-sensitive hydrogel that would be forwarded towards studying encapsulated CDC survival. This gel was characterized by composition by nuclear magnetic resonance and fourier transform infrared spectroscopy, lower critical solution temperature by differential scanning calorimetry, elastic modulus by uniaxial tensile testing, biodegradability in water, and catheter injectability. A series of pH- and thermal responsive hydrogels were developed by free radical polymerization of n(isopropylacrylamide), propylacrylic acid, 2-hydroxyethyl methacrylate-co-trimethylene carbonate.

1. Introduction:

1.1 Background

Cardiovascular disease is the leading cause of death in the U.S., accounting for approximately one in four U.S. deaths each year and affecting 83.6 million U.S. adults each year [1,2]. Furthermore, cardiovascular disease is projected to affect 40.8% of the U.S. population by 2030[3]. Of the conditions associated with cardiovascular disease (CVD), acute myocardial infarction (MI) is propagated through regional blockage of coronary arteries and impaired supply of oxygen and nutrition to areas of heart, leading to the failure and loss of heart cells that uphold normal heart functionality (**Fig. 1**). These lost cells cannot be regenerated by the body spontaneously and subsequently hinder normal cardiac function, resulting in death of heart tissue, and eventually heart failure [4]. While interventional procedures such as balloon angioplasty and stent placement seek to reduce the myocardial stresses coinciding with and onset by MI by restoring normal blood flow, they are deficient in restoring cardiac function to infarcted areas of the heart [5].

Figure 1: Anatomy of myocardial infarction.



Stem cell therapy is a promising method to cure MI through delivery of stem cells into the heart in order to regenerate lost myocardium. Delivered stem cells are recognized for their potential for full recovery of heart function, of which cardiosphere-derived stem cells (CDCs) are of particular interest for their capacity to

differentiate into new myocardium and endothelium - both *in vivo* and *in vitro* - and retain a high proliferation rate [6]. Unfortunately, current stem cell therapies, including direct injection of CDCs, experience low cell retention and survival resultant from the lack of matrix anchorage sites and harsh microenvironment of the infarcted area [7]. Encapsulated CDC delivery has been accomplished through open-heart surgery, but this method is time-sensitive and leaves the patient prone to post-operative infection. Thus, a means to improve efficacy of stem cell therapy that would counteract low cell retention and engraftment, and provide a stable environment for stem cells to differentiate and reestablish lost heart tissue applying minimally-invasive methods is proposed to circumvent associated risks.

1.2 Hydrogels

Hydrogels are unique delivery vehicles for tissue engineering and regenerative medicine. Hydrogels are water-swollen, cross-linked networked polymeric structures held together through hydrogen bonding and van der Waal inter-chain interactions [8]. Hydrogels are exceptional candidates for application in stem cell therapy due to their capacity to establish a stable biochemical and biomechanical environment, like that of typical cardiac conditions, while sustaining stem engraftments [9]. Specifically, thermoresponsive hydrogels are particularly effective for their solidification upon injection that would allow cells to physically bind and establish a matrix for stem cell adhesion [10]. The family of n-isopropylacrylamide-based (NIPAAm) thermoresponsive hydrogels have gained significant attention for increased cell retention consequent from fast gelation *in situ* at near core body temperature, improved cell survival and capacity to stimulate stem cell differentiation by

providing a suitable matrix. As these gels are thermally sensitive, the hydrogels may be delivered through minimally-invasive catheter application. These NIPAAm hydrogels are not readily applicable to stem cell therapy. Previously developed gels cannot be applied in catheter based percutaneous coronary intervention delivery because they are thermal-sensitive only and would solidify prematurely within the near-body temperature (37^{o}C) catheter, blocking any additional flow. Thus, a critical aim of this project addresses this situation by adjusting a pH sensitivity component to the polymer such that it would respond to the infarcted heart's environmental pH of 6.5-6.8 [11]. This would allow continued flow of the hydrogel within the catheter but allow for rapid gelation in the afflicted region. Thus, modifications to the NIPAAm polymer must retain particular thermal and mechanical properties while negotiating pH-sensitivity in order to effectively accommodate the shortcomings of thermal sensitive hydrogels.

Poly(n-isopropylacrylamide) (PNIPAAm) is a thermoresponsive hydrogel that exhibits a lower critical solution temperature at 32^{o}C. PNIPAAm thus forms a hydrogel at temperatures greater than 32^{o}C yet stays in solution at temperatures lower than 32^{o}C, within proximity of core body temperature [12].

Propylacrylic acid (PAA) is a monomer characterized by an ionizable carboxyl group that lends PAA its pH-sensitivity [13]. Previous research indicates that a PAA-based hydrogel is able to maintain a solution state at pH 7.4 at body temperature and at lower pH at 5^{o}C, but showed rapid gelation at low pH and at nominal body temperature [9]. This indicates an ideal candidate for minimally-invasive delivery of the hydrogel and rapid gelation on MI sites.

Hydroxyethyl methacrylate-poly(trimethylene carbonate) (HEMA-PTMC) is a biocompatible polymer that may adjust the mechanical properties of the hydrogel to achieve characteristics ideal for cell survival based upon varying molar ratios of NIPAAm and HEMA-PTMC [14]. Such characteristics may be adjusted to match mechanical properties similar to that of the heart, such as elasticity and dynamic motion. The objective of this project is to further develop and characterize hydrogel copolymer of various monomer ratios of NIPAAm, PAA, and HEMA-PTMC suitable for cardiosphere-derived stem cell delivery. These hydrogels should retain tunable thermal, mechanical, and pH-sensitive properties that enable the gel to function in cardiac microenvironments, yet display a degree of degradability, injectability, and biocompatibility.

2. Materials and Methods

2.1 Materials

N-isopropylacrylamide and 2-hydroxyethyl methacrylate (HEMA) were purchased from VWR. NIPAAm was recrystallized three times with hexane. HEMA was purified by passing the HEMA through an inhibitor remover-packed column. Trimethylene carbonate (TMC) was purchased from Boehringer Ingelheim and was used without purification. Diethyl propylmalonate and diethylamine were purchased from Alfa Aesar and were used without purification. Formalin solution was purchased from Fischer Scientific and used without purification. Benzoyl peroxide (BPO) and other relevant solvents were used as received from VWR.

2.2 Synthesis of Propylacrylic Acid

Propylacrylic acid was synthesized following methods adopted from Ferritto and Tirrell [16] (**Fig. 2**). 25 g diethyl propylmalonate was used without purification and was stirred in 175 mL 1 M KOH 95% ethanol solution for 18 hours. The mixture was condensed by rotary evaporator at 0^{o}C and dissolved in a minimal amount of deionized water. The solution was acidified with 1 M hydrochloric acid to adjust to a pH of 2. The crude product was extracted with three times with 200 mL ethyl ether, dried by magnesium sulfate overnight, and filtered. Excess ether was removed by rotary evaporator. The structure of 2-carboethoxypentanoic acid was confirmed by ^{1}H-NMR. The oily product was reacted with 12.8 mL diethylamine at -5^{o}C and allowed to homogenize. 12.3 mL formalin solution was slowly added by addition funnel and allowed to stir for 24 hours. The mixture was then allowed to reflux for 8 hours at 60^{o}C. The mixture was cooled to 0^{o}C and concentrated

sulfuric acid was added until violent reaction ceased. The crude product was extracted with three times with 200 mL ethyl ether, dried by magnesium sulfate overnight, and filtered. The structure of the crude ethyl 2-propylacrylate was confirmed by ^{1}H-NMR. The crude product was hydrolyzed by 1 M KOH and refluxed for 20 hours. The solution was acidified to adjust the product to pH 2. A yellow oil was separated and extracted with 200 mL ethyl ether diethyl ether three times, dried by magnesium sulfate overnight, and filtered. Excess ether was removed by rotary evaporator.

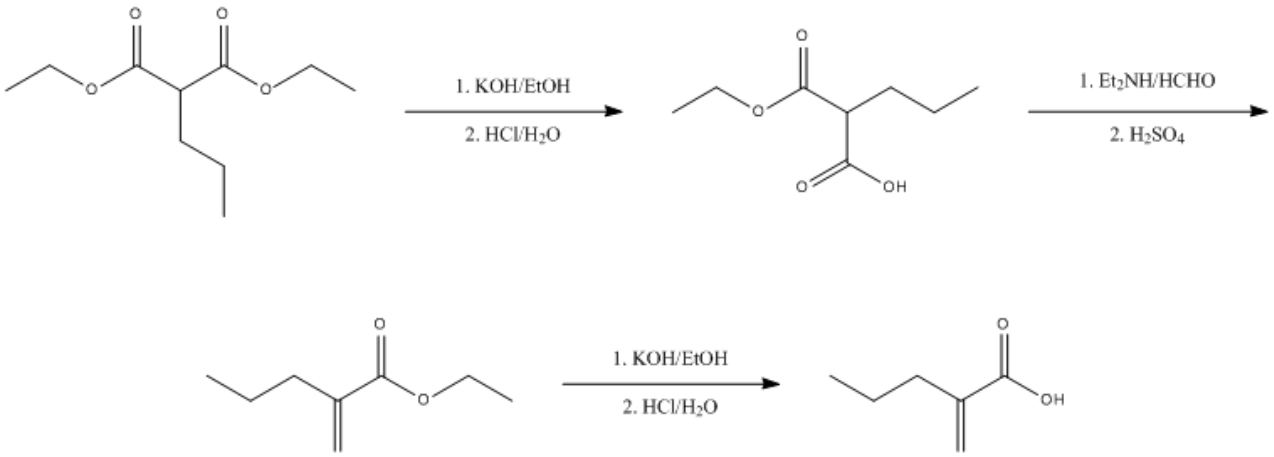


Figure 2: Synthesis reaction schematic of propylacrylic acid

2.3 Synthesis of hydroxyethyl methacrylate-poly(trimethylene carbonate)

Hydroxyethyl methacrylate-poly(trimethylene carbonate) (HEMA-PTMC) was synthesized by reacting HEMA with TMC with a molar ratio of 1 to 2 respectively at 110^{o}C for 1 hour with stannous octoate catalyst (**Fig. 3**) [9,10]. The crude product was dissolved in tetrahydrofuran (THF) and precipitated in 0^{o}C deionized water. The mixture was centrifuged and the supernatant was poured off. The precipitate was re-dissolved in ethyl acetate and dried by magnesium sulfate. The product was condensed by rotary evaporator.

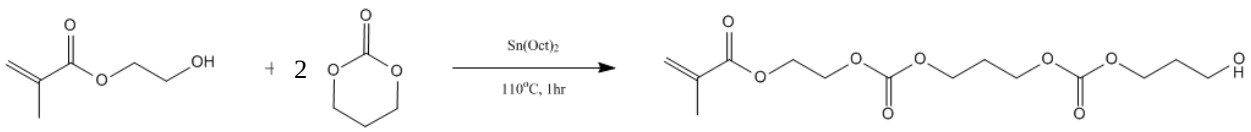


Figure 3: Synthesis reaction schematic of HEMA-PTMC

2.4 Synthesis of poly(NIPAAm-co-PAA-co-HEMA-PTMC)

NIPAAm, PAA, and HEMA-PTMC were reacted in varying molar ratios (**Table 1**) by free radical polymerization (**Fig. 4**) to obtain hydrogels with varying pH and mechanical properties. The reactants were dissolved in dioxane at 25°C, and the reaction flask was flushed with nitrogen gas for 15 minutes. The initiator benzoyl peroxide (BPO) was added and the reaction was maintained at 70°C for 20 hours. The solution was precipitated into hexane to obtain a white solid. The mixture was filtered and dissolved in tetrahydrofuran (THF). The crude product was then precipitated and by purified by THF and diethyl ether two times, then freeze-dried overnight.

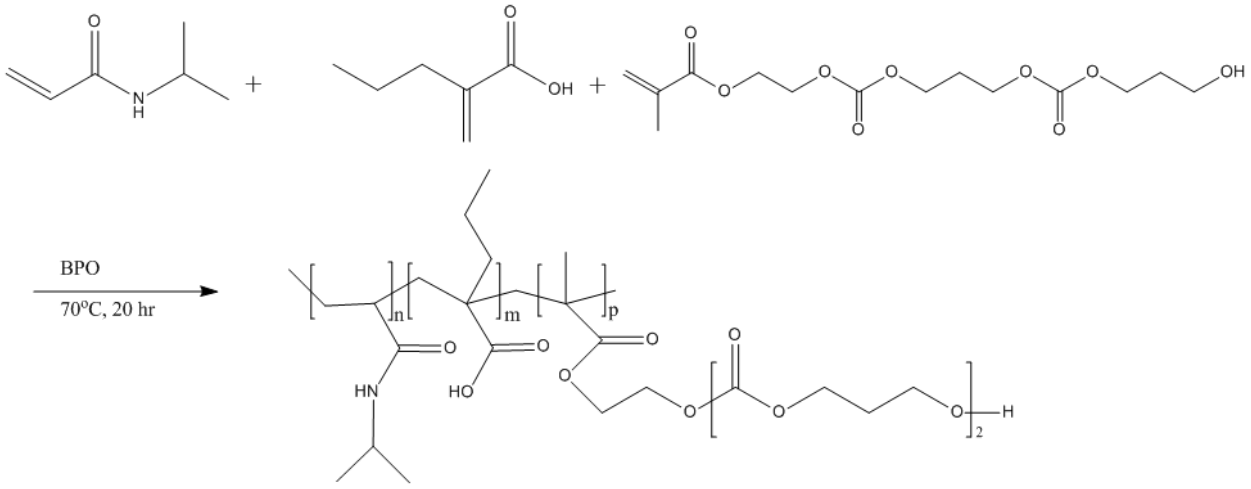


Figure 4: Synthesis reaction schematic of final product

Table 1: Monomer molar feed ratio (%) (NIPAAm/PAA/HEMA-PTMC)

Hydrogel	Feed Ratio
Gel 1	84/8/8
Gel 2	83/12/5
Gel 3	78/12/10

2.5 Hydrogel Characterization

20 % wt. hydrogel solution was prepared by dissolving dried copolymer in Dulbecco's phosphate-buffered saline (DPBS) at 0°C. Sodium hydroxide was used to adjust the pH of the dissolved hydrogel samples for differential scanning calorimetry and catheter injectability tests to pH 6.5 and 7.4. The pH of the hydrogel solutions were not adjusted for all other analyses. Composition of the hydrogels and functional groups were determined by nuclear magnetic resonance and fourier transform infrared spectroscopy.

2.5.1 Hydrogel Water Degradation

200 µL of 20 wt. % hydrogel solution was added to a 1.5 mL Eppendorf tube and allowed to gelate in a 37°C water bath for 24 h. The supernatant was discarded and replaced with 1 mL of 37°C DPBS and the pellets were allowed to incubate at 37°C. Hydrogel water degradation was conducted over an eight week time period with samples collected at intervals in weeks 0, 1, 2, 3, 4, 6, 8. The hydrogel was freeze-dried at each time point and the remaining gel pellet was weighed in the interval progression, and the content loss was used to report weight remaining. The weight remaining was used to quantify the degradation of the hydrogels.

2.5.2 Stretchability

Hydrogel samples were allowed to solidify at 37°C for 1 hour to form solid pellet. The measured sample was sectioned to obtain a sample of 3.5 mm width, stretched in 37°C water bath, and its final length was observed.

2.5.3 Tensile Test

Elastic modulus of the hydrogels was determined via tensile testing. Hydrogel samples were incubated at 37^{o}C for 1 hour to form solid pellet and then sectioned into 4 samples each approximately 3.5 mm width and 20 mm length. The tensile test was conducted in a 37^{o}C water bath. A cross-head speed of 50 mm/min was used.

2.5.4 Catheter Injectability

Injectability was examined by injecting 4^{o}C pH 7.4 hydrogel solution into sodium phosphate buffers with pH of 6.5 and 7.4 at 37^{o}C through a 26-gauge catheter (Abbot Voyage).

2.5.5 Differential Scanning Calorimetry (DSC)

The thermal properties of these hydrogel solutions at pH 6.5 and 7.4 were determined by differential scanning calorimetry over a temperature range from 0^{o}C to 60^{o}C with a 10^{o}C/min heating rate. The temperature at the maximum endothermal peak was used as the lower critical solution temperature (LCST).

3. Results

3.1 Synthesis of propylacrylic acid, HEMA-PTMC, and poly (NIPAAm –co-PAA –HEMA-PTMC)

The structures of propylacrylic acid, HEMA-PTMC, and three copolymers were confirmed by ^{1}H-NMR. The structure of PAA was confirmed by ^{1}H-NMR by chloroform, δ: 6.23 (1H,s, CH2=), 5.58 (1H, s, CH2=), 2.22 (2H, t, -CH2-), 1.44 (2H, m, -CH2-) and 0.87 (3H, m, CH3-). The final product was a yellow oil. The spectra of PAA, HEMA-PTMC, and poly(NIPAAm – co – PAA – HEMA-PTMC) (Gel 1) are shown in (**Fig. 5.1**-**5.3**). The Gel 1 is presented as a representative. Characteristic peaks were clearly observed in the spectra, indicating the desired structures were obtained. The average unit ratio between TMC and HEMA was calculated based upon the NMR spectrum to be 2.07. The copolymer composition was determined by the calculation of the ratios of the characteristic peak integrals of NIPAAm, PAA, and HEMA-PTMC. The composition ratios of the copolymers were found to be different from the feed ratios (**Table 2**). All the characteristic peaks were observed in the NMR spectrum for the synthesized copolymers: HEMA-PTMC (l,q), NIPAAm (d), PAA (i).

Table 2: Copolymer composition ratio (%) (NIPAAm/PAA/HEMA-PTMC)

Hydrogel	Feed Ratio	Actual Ratio
Gel 1	84/8/8	70/13/17
Gel 2	83/12/5	71/17/12
Gel 3	78/12/10	62/18/20

Figure 5.1: ^{1}H-NMR spectrum of propylacrylic acid monomer

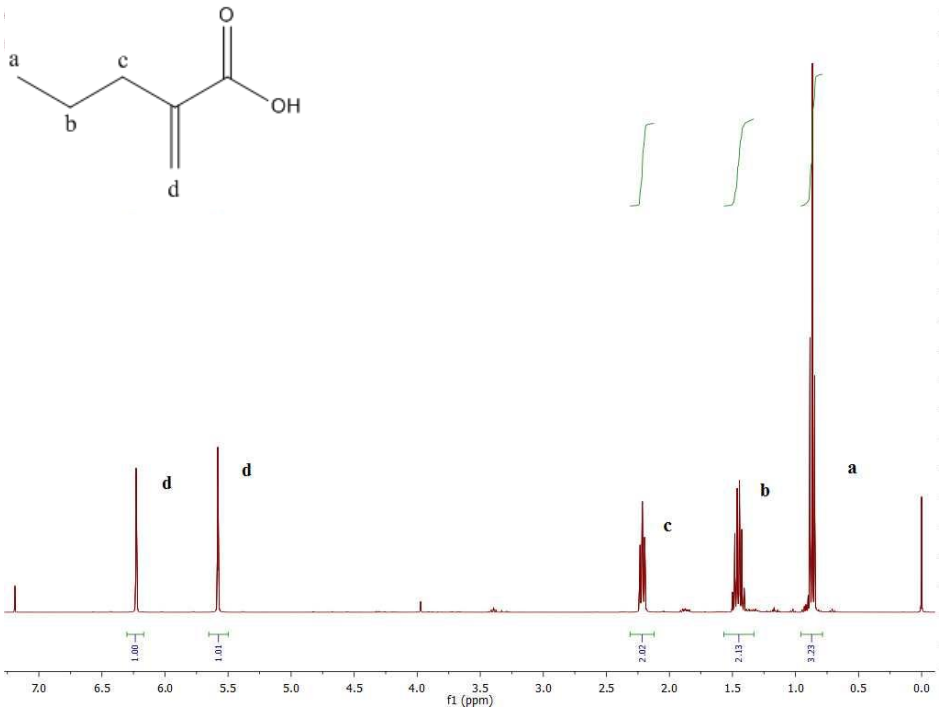


Figure 5.2: ^{1}H-NMR spectrum of HEMA-PTMC

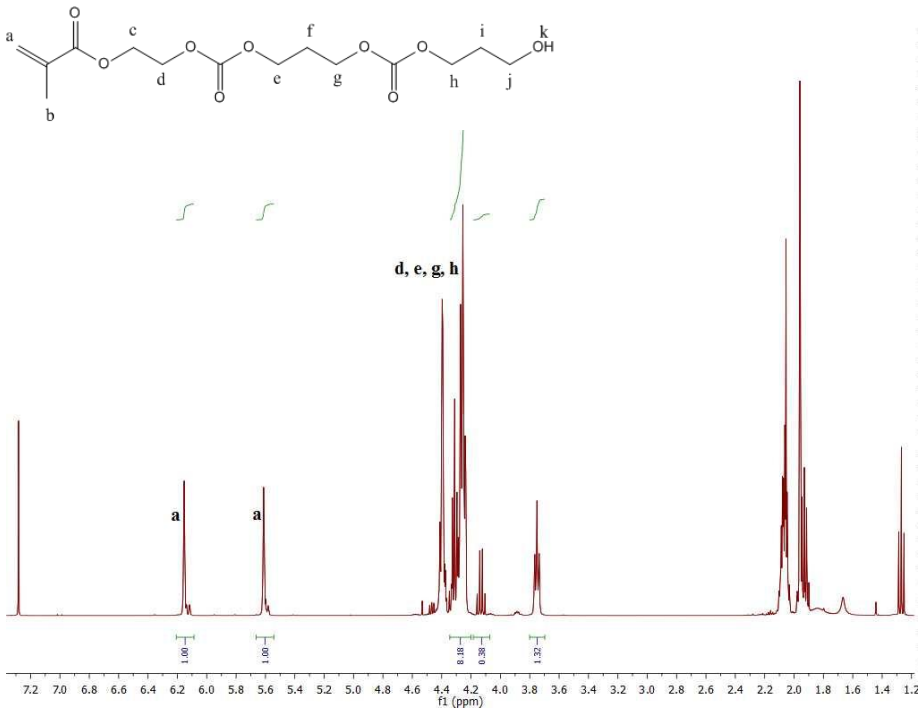
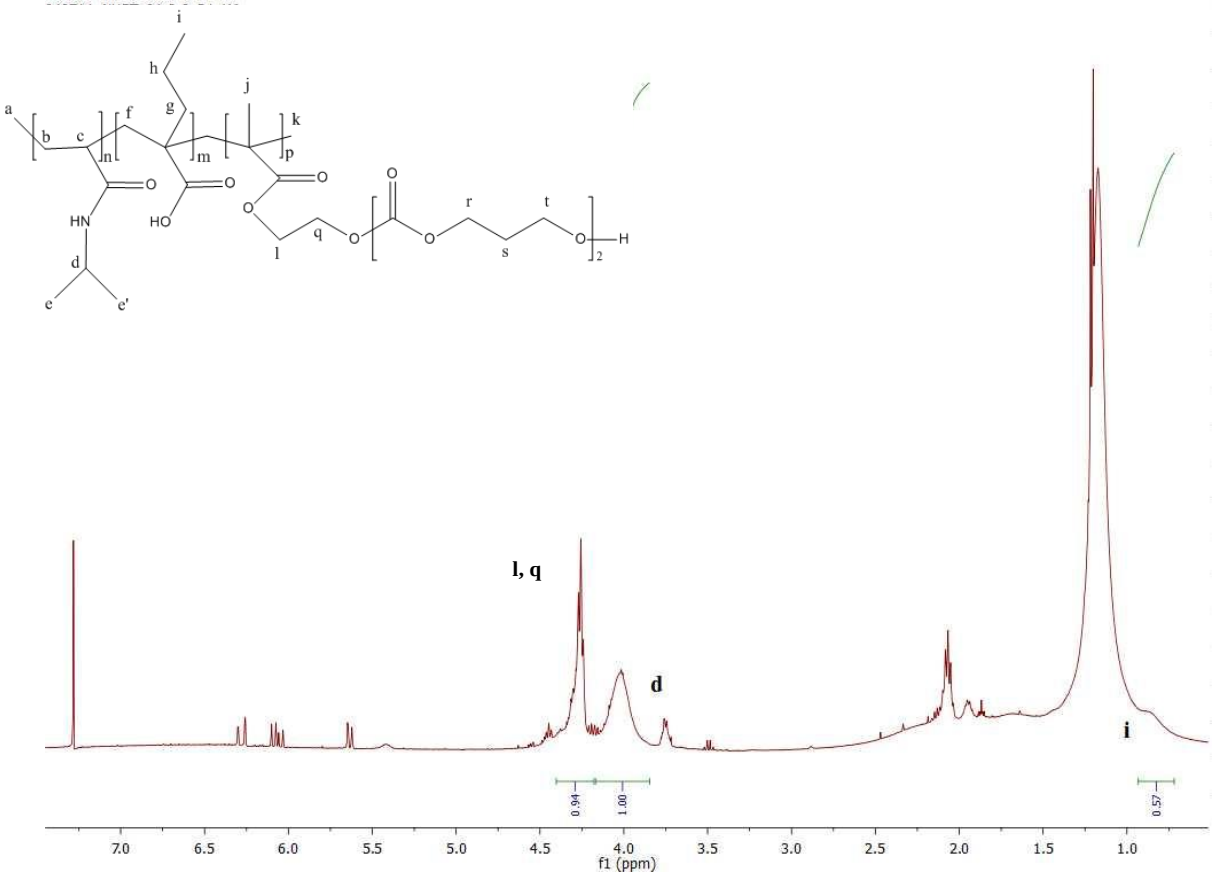
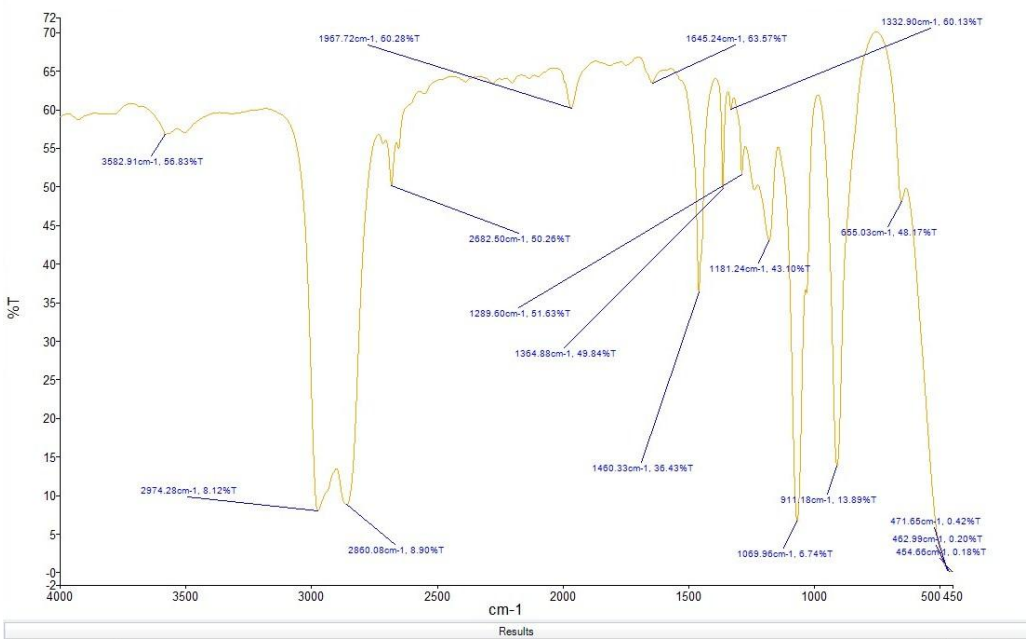


Figure 5.3: ^{1}H-NMR spectrum of poly(NIPAAm-co-PAA-HEMA-PTMC) copolymer (Gel 1)



FTIR spectra indicates that all hydrogels possessed characteristics absorptions of NIPAAm, PAA, and HEMA-PTMC (**Fig. 6**). Gel 2 is shown as a representative.

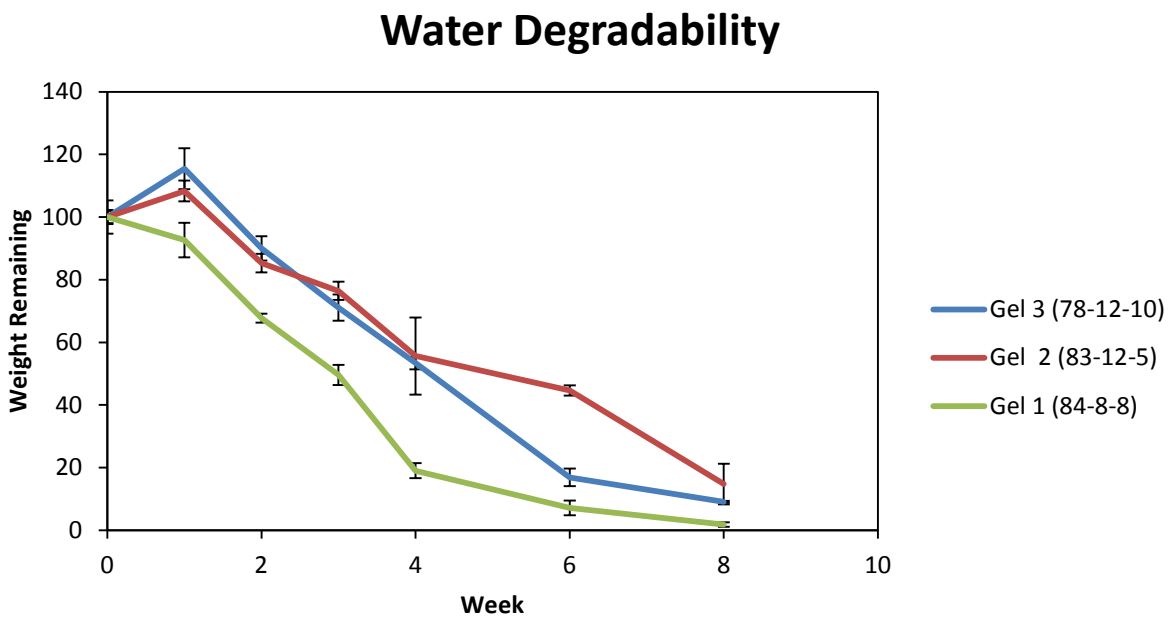
Figure 6: FT-IR spectrum of poly(NIPAAm-co-PAA-HEMA-PTMC) copolymer (Gel 2)



3.2 Hydrogel Characterization

All hydrogels demonstrated progressive degradation during the eight week period. Gels 2 and 3 retained above 50% of their weight by week 4, but Gel 1 demonstrated comparatively rapid content loss (**Fig. 7**).

Figure 7: Hydrogel biodegradation at 37°C incubation (% content remaining)



Gels 1 and 3 demonstrated they are flexible and can be stretched at 37°C under aqueous conditions. Gel 2, however, was too fragile and failed to demonstrate any degree of stretching (**Fig. 8**).

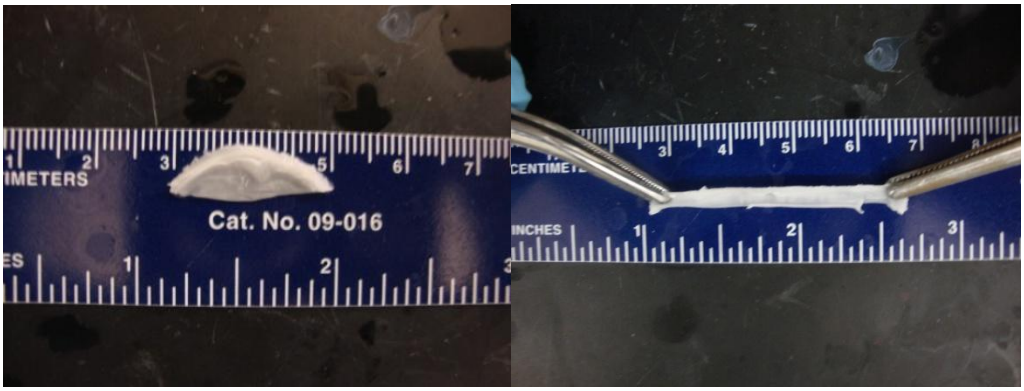


Figure 8: Gel 1 is flexible at 37°C and can be stretched (left) before stretching, (right) after stretching. Gel 3 demonstrated similar properties.

The mechanical properties of the hydrogel were tested using an Instron tensile tester for elastic modulus (**Table 3**). The initial linear region in the stress-strain curve was used to

calculate the hydrogel elastic modulus in Gels 1 and 3 via a Matlab program. Gels 1 and 3 displayed over 300% overall strain capacity. Gel 2 was too fragile to undergo the tensile test.

Table 3: Hydrogel elastic modulus at 37°C via tensile test

Hydrogel	Elastic Modulus (kPa)
Gel 1	64.60 ± 25.84
Gel 3	219.62 ± 63.45

3.3 Hydrogel Solution Characterization

As gelation is pH dependent, it was found that all gels could be readily injected through the catheter. Upon injection into the warm phosphate buffers, Gel 1 exhibited gelation in both pH 7.4 and 6.5 buffer. Gel 2 showed gelation pH 7.4 buffer, but also indicated gelation pH 6.5 buffer. Gel 3 retained its solution state in pH 7.4 sodium phosphate buffer, but showed gelation in 6.5. Injectability and gelation results for Gel 3 are displayed in **Fig. 9.1** and **9.2**. LCST of the hydrogel solutions were measured by DSC and are reported in **Table 4**. Only gel 1 did not achieve 37°C in the pH 7.4 conditions. Gel 2, however, exhibited an LCST greater than 37°C in acidic conditions. The relation of LCST and PAA is observed. The LCST of both environments increased with PAA composition.

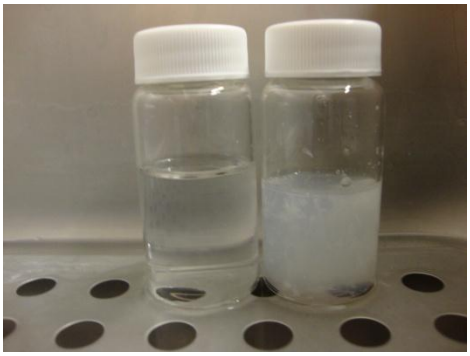


Figure 9.1: Adjusted pH 7.4 Gel 3 at 4°C injected into buffer pH 7.4 (left) and pH 6.5 (right) at 37°C

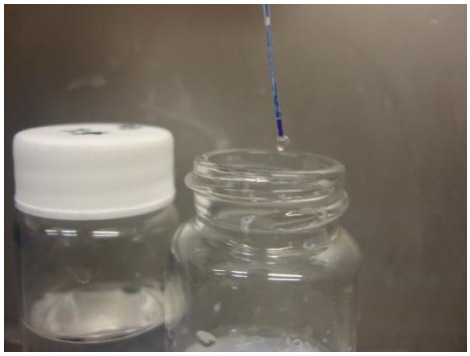


Figure 9.2: All gels were injectable through a 26-gauge catheter

Table 4: Hydrogel solution characterization

Hydrogel	Injectability	LCST (pH 6.5)	LCST (pH 7.4)
Gel 1	+	30.52°C	35.56°C
Gel 2	+	40.57°C	45.00°C
Gel 3	+	31.97°C	38.91°C

4. Discussion

A pH- and thermoresponsive injectable hydrogels has been proposed as an alternative to circumvent the issues of premature gelation during catheter-based PCI delivery. A series of pH- and thermoresponsive injectable hydrogels were synthesized through varying the molar feed ratios of PNIPAAm, PAA, and HEMA-PTMC.

4.1 Synthesis of PAA and HEMA-PTMC monomers and poly(NIPAAm-PAA-HEMA-PTMC) copolymers

As has been widely established, the temperature, NIPAAm's temperature sensitivity derives from hydrophilic and hydrophobic interactions with water in the isopropyl groups. Consequently, propylacrylic acid was integrated into the synthesis of the copolymers to introduce pH sensitivity to these thermosensitive hydrogels through capitalizing on similar hydrophilic and hydrophobic interactions. The results showed Gels 1 and 3 had LCSTs lower than 37^{o}C at pH 6.5 and could still be injected through a catheter. While all three gels displayed varying degrees of gelation at pH 6.5 in 37^{o}C aqueous environment after catheter injection, characteristic of ischemic conditions, gelation behavior of Gels 1 and 2 are inconsistent with the pH-sensitivity by also exhibiting gelation in pH 7.4 conditions. This indicates Gel 3 alone possesses ideal LCSTs for catheter-based delivery. HEMA-PTMC was introduced to alter the mechanical properties of the hydrogel, and the introduction of HEMA-PTMC increases the hydrophobic interactions leading to a lower LCST. Gels 1 and 3 had greater relative HEMA-PTMC content and possessed comparatively lower LCSTs. The biodegradability of the polymers suggests the greater PAA concentration in the copolymer

increases the hydrophilicity of the polymer as indicated by the lower content remaining in Gel 3 relative to gels 1 and 2.

4.2 Mechanical Properties of Copolymer Hydrogels

The mechanical properties of hydrogels were observed to be dependent on copolymer composition. The absence of elastic modulus and stretching data for Gel 2 attributed to its brittleness brings into question the opportunity for cross-linking during the polymerization process. This could account for the outstanding behavior of Gel 2 in comparison with that of Gels 1 and 3. Simultaneously, the elastic modulus and stretchability for Gels 1 and 3 indicate a greater HEMA-PTMC concentration within the hydrogel (**Table 2**) may increase the elastic properties of the polymer. This is supported by the greater elastic modulus of Gel 3 than that of Gel 1. In conjunction with the injectability results, it appears the greater hydrophobicity of the hydrogel coincides with greater elastic modulus by way of increased PAA and HEMA-PTMC.

5. Conclusions

A series of pH- and thermo-sensitive poly(NIPAAm-co-PAA-co-HEMA-PTMC) hydrogels (Gels 1, 2, and 3) were synthesized by free radical polymerization. All three gels displayed injectability through a catheter at 37^{o}C, and Gel 3, feed ratio 78/12/10, showed promising LCSTs in both pH 6.5 and 7.4 environments. Gel 3 showed optimal mechanical properties and degradation behavior over an 8-week period. It may be concluded that Gel 3 may provide a potential cell delivery vehicle for stem cell therapy.

Future Work:

There are inconsistencies in the data that would not be able to substantiate many conclusions in the absence of determining the polymer's molecular weight. In this regard, all three hydrogels will be submitted for gel permeation chromatographic analysis to determine hydrogel molecular weight and polydispersity index. The calculated composition ratios may also suggest an alternative copolymer synthesis mechanism such as reversible addition-fragmentation chain-transfer polymerization may offer resolution to this situation [13].

CDC encapsulation in the hydrogels to quantify cell survivability would evidence the viability of these hydrogels as a stem cell therapy vehicle, and to observe which of the gels and their respective properties would confer the fitting composition for this objective. This biocompatibility analysis would provide insight into the biological capacities of the hydrogel copolymers.

Finally, a degradation series should be examined in respective pH 6.5 and 7.4 as would be representative of the cardiac microenvironments.

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